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Desmosomes are intercellular junctions that confer strong cell–cell adhesion. Altered expression of desmocollin 3 (DSC3), a member of the desmosomal cadherin family, was found in various cancers; however, its functional involvement in carcinogenesis has not yet been elucidated. Expression/localization of DSC3 was analyzed by real-time reverse transcription–PCR, western blotting, immunofluorescence and immunohistochemistry. Methylation status of DSC3 was examined by demethylation tests, methylation-specific PCR and bisulfite sequencing. It turned out that downregulation of DSC3 in lung cancer cells was associated with DNA hypermethylation. In primary lung tumors, DSC3 was a potential diagnostic marker for lung squamous cell carcinoma, and DSC3 DNA hypermethylation was correlated with poor clinical outcome. To investigate the effect of the tumor suppressor gene p53 on DSC3, transient transfection with a wild-type p53-expression vector was performed. Overexpression of p53 resulted in an increased expression of DSC3 in a DSC3-unmethylated lung cancer cell line H2170, but not in H1299, a DSC3-methylated cell line. However, combination of p53 transfection with demethylation agent 5-aza-2’-deoxycytidine treatment led to increased expression of DSC3 in H1299 cells. Furthermore, functional studies after stable transfection of a DSC3 expression vector showed that ectopic expression of DSC3 inhibited cell proliferation, anchorage-independent growth, migration, as well as invasion, and most interestingly led to reduced phosphorylation levels of extracellular signal-regulated kinase 1/2. Taken together, our data suggested that DSC3 acts as a novel tumor suppressor gene through inhibition of epithelial growth factor receptor/extracellular signal-regulated kinase signaling in lung cancer cells.

Introduction

Lung cancer is the most commonly diagnosed cancer, accounting for almost 18% of all cancer-related deaths worldwide in 2008 (1). Local invasion and distant metastasis are implicated in the pathophysiology of lung cancer and cause the major determinant of poor outcome with an overall 5-year survival rate of 15% for all stages (2). Despite advances in understanding the molecular pathogenesis of invasion and distant metastasis, our knowledge on the mechanisms remains incomplete.

In desmosomes, members of the cadherin family represent the major adhesive cell junctions of epithelial cells (3,4). Recent studies suggest that downregulation of desmosomes may contribute to malignant progression (5). For example, the loss or reduction of one or more desmosomal components, including DSG1-3 and DSC2, was observed during development and/or the progression of various human epithelial cancers, including uterine endometrial, skin, gastric, colon, and head and neck cancers (6–10).

Desmocollin 3 (DSC3), a member of cadherin superfamily of calcium-dependent cell-adhesion molecules, contributes to desmosome-mediated cell–cell adhesion. Recent studies indicate that loss of DSC3 is involved in several malignancies. Oshiro et al. (11) reported that DSC3 was downregulated in breast cancer due to promoter hypermethylation. Expression of DSC3 was reduced in lymph node metastases of oral squamous cell carcinoma (SCC) (12). In addition, our previous study revealed the loss of DSC3 by epigenetic regulation in colorectal cancer (13). In lung cancer, the diagnostic value of DSC3 in squamous carcinoma has been reported (14,15); however, little is known about the regulatory mechanisms and functional role of DSC3 in this respect.

The epidermal growth factor receptor (EGFR) plays an important role in modulating intercellular adhesion, especially in tumors overexpressing this tyrosine kinase. Overexpression of EGFR was found in 50–70% of human lung cancer (16), and EGFR tyrosine kinase inhibitor gefitinib (Iressa) recently has been considered first-line therapy for patients with advanced non-small-cell lung cancer. The role of EGFR pathway in regulating tumor cell proliferation, apoptosis and survival has been well elucidated; however, little attention has been paid to the interplay between EGFR and cell adhesion in lung cancer cells.

In this study, we observed that the loss of DSC3 in human lung cancer is associated with DNA hypermethylation. DSC3 is regulated by p53 and DSC3 exerts its tumor-suppressive activity through inhibition of the EGFR/extracellular signal-regulated kinase (ERK) signaling pathway in lung cancer cells.

Materials and methods

Cell lines and cell culture

Human bronchial epithelial cells (HBECs) were obtained from Clonetics (San Diego, CA, USA) and cultured in BEG media (Lonza, Walkersville, USA). Eleven lung cancer cell lines, including seven non-small-cell lung cancers (H23, H2030, H2228, H157, H226, H2170, H1299) and four small-cell lung cancers (SHP77, H82, COLO668, COLO677), were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). Cells were grown in RPMI 1640 medium (Biochroon AG, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum and maintained in a humidified atmosphere with 5% CO₂ at 37°C.

For drug treatment, cells were starved overnight and then treated with 10 µM gefitinib (LC laboratory, Woburn, MA USA) for 24h or 50 µM ERK inhibitor PD98059 (LC laboratories) for 60 min, and cells were further incubated with 50 ng/ml of EGFR (Sigma Chemical Co., St Louis, MO USA) for 30 min.

RNA extraction and real-time RT–PCR

RNA extraction was carried out as previously described (13). Five hundred nanograms of total RNA was reverse transcribed into complementary DNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR was performed in 0.1 ml tubes on the Rotor-Gene 6000 (Qiagen) in the presence of the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). Primer sequences are shown in Supplementary Table S1, available at Carcinogenesis Online. The relative expression value of DSC3 to glyceraldehyde 3-phosphate dehydrogenase in each sample was calculated and compared. The experiments were performed in triplicates.

Western blot analysis

Proteins from whole cell lysate were isolated as described previously (13). Ten micrograms of protein were used for western blotting according to standard protocols. The information about the antibodies applied for western blotting is given in Supplementary Table S2, available at Carcinogenesis Online. Signals
were visualized with horseradish peroxidase–conjugated polyconal rabbit antinoucleo antibody or goat antirabbit antibody and ECL Plus Western blotting Detection System (GE Healthcare, Munich, Germany).

**Immunofluorescence**

Cells were grown on 4-well culture slides (BD Biosciences, Heidelberg, Germany) in RPMI 1640. After 24 h, culture cells were fixed with -20°C acetone for 3 min. After air drying, the slides were immediately subjected to immunofluorescence staining. Cells were permeabilized in phosphate-buffered saline + 0.3% (v/v) Triton X-100, blocked with phosphate-buffered saline + 5% (v/v) normal goat serum for 1 h. Subsequently, cells were incubated with primary antibody overnight at 4°C, and after a washing step in phosphate-buffered saline, fluorescence-labeled secondary antibody was added for 1 h (Supplementary Table S2, available at Carcinogenesis Online). Coverslips were washed and mounted using Mounting Medium for fluorescence (Vector Laboratories, Burlingame, CA USA). Samples were analyzed under an Axio Observer Z1 microscope (Carl Zeiss Microimaging GmbH, Jena, Germany).

**Demethylation tests**

For demethylation tests, 11 lung cancer cell lines were plated and cultured on six-well plates. At 50% confluence, 10 μM DAC (Sigma Chemical Co., St Louis, MO USA) was added to the medium on days 0, 2, and 4. Cells were harvested for total RNA isolation and real-time reverse transcription (RT)–PCR analysis.

**Bisulfite modification, methylation-specific PCR and bisulfite sequencing**

Bisulfite modification, methylation-specific PCR (MSP) and bisulfate sequencing (BS) were carried out as described previously (13). Primer pairs for MSP and BS (Supplementary Table S1, available at Carcinogenesis Online) were designed to determine the methylation pattern of the CpG sites in DSC3 promoter region. Primers used for BS did not contain any CpG site in their sequences. Primers used for BS did not contain any CpG site in their sequences, so both methylated and unmethylated sequences can be amplified, and the methylation status was determined after DNA sequencing. For MSP, two pairs of primers were used to amplify methylated or unmethylated sequences.

**Transient transfection**

To explore the effect of p53 on DAC-induced expression of DSC3, H1299 cells (1 × 10⁵ cells/well in 12-well plates) were transfected with 5 μM DAC on days 0 and 2. On day 3, cells were transfected with a wild-type p53 expression vector (gift from Dr Ying Liu, Oxford University, UK) using FuGENE HD Transfection Reagent (Roche Applied Science, Mannheim, Germany) and incubated for another 48 h.

**Primary lung tumors and genomic DNA isolation**

A total of 80 tumor specimens (paraffin tissues) from patients with primary lung cancer were included in the study (Table I). All of the patients were undergoing surgical operation at the Department of Surgery of the University Hospital Charité from 1995 to 2000. No adjuvant radiotherapy or chemotherapy was administered before surgery. The study was approved by the local ethical committee. All hematoxylin and eosin sections were reviewed by two pathologists (I. Petersen and T. Knösel). Suitable areas for genomic DNA isolation were marked on hematoxylin and eosin sections. Genomic DNA was extracted as described previously (13).

**Tissue microarray construction and immunohistochemistry**

Tissue microarray (TMA) was constructed using a manual tissue arrayer purchased from Beecher Instruments (Woodland, WI, USA). Two tissue cylinders per tumor with a diameter of 0.6 mm were present in one TMA.

<table>
<thead>
<tr>
<th>Clinicalpathologic feature (number of cases examined)</th>
<th>Number of cases</th>
<th>DSC3 expression</th>
<th>p*</th>
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<tbody>
<tr>
<td>Methylation status (80)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Methylated</td>
<td>34</td>
<td>29 (36.3)</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>46</td>
<td>28 (35.0)</td>
<td>18 (22.4)</td>
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<tr>
<td>Non-small-cell lung cancer (60)</td>
<td></td>
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<tr>
<td>SCC</td>
<td>22</td>
<td>8 (13.3)</td>
<td>14 (23.4)</td>
</tr>
<tr>
<td>ADC</td>
<td>38</td>
<td>35 (58.3)</td>
<td>3 (5.0)</td>
</tr>
</tbody>
</table>

The values in parenthesis are expressed in percentage.

*P < 0.05 was considered statistically significant.

**Statistical analysis**

The statistical analysis was performed using the software package SPSS 19.0 (SPSS, Chicago, USA). Student’s t-test was carried out to detect the differences between DSC3 transfectants and control cells. Chi-square or Fisher’s exact test was performed to analyze the association between DSC3 protein expression/methylation and clinicopathological parameters. Kaplan–Meier survival curves were constructed for statistical significance with the log-rank tests. P values were calculated two-sided. P values less than 0.05 were defined as statistically significant.
Results

Loss of DSC3 expression in lung cancer cell lines
To analyze the expression of DSC3 in 11 lung cancer cell lines together with HBEcs, real-time RT–PCR and western blotting were performed. Compared with HBEc, DSC3 mRNA expression was strongly reduced in all the lung cancer cell lines (Figure 1A). This result was consistent with western blotting (Figure 1B) demonstrating decreased expression of DSC3 in lung cancer cell lines.

Methylation of DSC3 DNA in lung cancer cell lines
To explore the epigenetic regulation of DSC3, all the 11 lung cancer cell lines were assayed by demethylation tests. After treatment with 10 µM DAC, a remarkably increased DSC3 mRNA expression was observed in five cell lines (H2030, H1299, SHP77, COLO677 and COLO668). The expression of DSC3 was slightly upregulated in two cell lines (H23 and H2228), whereas no restoration of DSC3 expression was detected in the other four cell lines including H157, H226, H2170 and H82 (Figure 1C).

To evaluate the detailed methylation pattern of DSC3 in lung cancer cells, BS was performed. In analysis of 21 CpG sites (-275, -272, -269, -260, -250, -224, -219, -215, -210, -204, -201, -199, -190, -183, -160, -150, -142, -138, -136, -132 and -128) of the promoter region, we detected DSC3 DNA hypermethylation in H2030, H1299, SHP77, COLO677 and COLO668. In H2228, DSC3 DNA was partially methylated, and in H23, the DSC3 DNA was very slightly methylated, whereas in H157, H226, H2170 and H82, DSC3 DNA was unmethylated (Figure 1D). As expected, DSC3 DNA was not methylated in normal control HBEC. These results were in good agreement with the demethylation tests.

The methylation status of the DSC3 promoter region was further determined by MSP. MSP showed that DSC3 was methylated in cell lines H2030, H2228, H1299, SHP77, COLO677 and COLO668, but unmethylated in cell lines H23, H157, H226, H2170, H82 (Supplementary Figure S1A, available at Carcinogenesis Online).

The reliability of the MSP results was verified by DNA sequencing (Supplementary Figure S1B, available at Carcinogenesis Online).

These results suggest that DSC3 DNA methylation is a frequent molecular event in lung cancer cells. The utility of the MSP primer tested in cell lines encouraged us to analyze the methylation status of DSC3 DNA in primary lung tumors.

Loss of DSC3 in primary lung cancer and methylation of DSC3 promoter DNA predicts poor clinical outcome
We analyzed the DSC3 protein expression in 80 primary lung tumors by IHC. It turned out that 23 out of 80 cases (28.7%) were positive for DSC3 with membrane staining (Figure 2A), whereas other 57 cases (71.3%) exhibited no expression of DSC3 (Table I). Since the sample size of small-cell lung cancer and large-cell lung cancer was not big enough, we only included SCC and adenocarcinoma for statistical analysis. Interestingly, SCC showed significantly more staining of DSC3 (14 out of 22, 63.6%, Table I) compared with adenocarcinoma (3 out of 38, 7.9%, Table I). However, the expression of DSC3 protein was not significantly linked to tumor stages, grades and clinical outcome (data not shown). Methylation of DSC3 promoter DNA was detected in 34 out of 80 primary lung tumors (42.5%, Table I) by MSP. Examples of MSP are shown in Supplementary Figure S1C, available at Carcinogenesis Online.

We further analyzed the correlation between DSC3 protein expression and DSC3 DNA methylation and found that decreased DSC3 protein expression was significantly correlated with DSC3 DNA methylation ($P = 0.017$, Table I), indicating that downregulation of DSC3 could be explained by DNA methylation in primary lung tumors. Finally, we investigated the effect of methylation on clinical outcome by Kaplan–Meier analysis. It turned out that DSC3 DNA methylation was significantly correlated with a worse overall survival (Figure 2B, $P = 0.048$).

Effect of p53 on regulating DSC3 mRNA expression
In breast cancer cells, p53 was reported to be an upstream factor that could regulate the expression of DSC3 (18). To investigate a potential link between p53 and DSC3 expression in lung cancer cells, we selected two cell lines H2170 and H1299 for transient transfection with a wild-type p53 expression vector. A successful transfection was confirmed by western blotting demonstrating the re-expression of p53 in both cell lines (Figure 2B, $P = 0.048$). Exogenous expression of p53 alone induced the mRNA expression of DSC3 in H2170 but not in H1299 (Figure 3B). We therefore analyzed the methylation status of DSC3 DNA in the
p53 binding site. It turned out that DSC3 DNA was unmethylated in H2170 but was highly methylated in H1299 (Figure 3C). Transfection of p53 in combination with the demethylation agent DAC treatment resulted in upregulation of DSC3 expression in H1299 (Figure 3D). These results suggest that expression of DSC3 is regulated by p53 in lung cancer cells depending on the methylation status of the DSC3 DNA in the p53 binding site.

**DSC3 inhibits tumor cell proliferation, clonogenicity, migration and invasion**

To explore the function of DSC3 in lung cancer cells, we constructed an expression vector encoding the wild-type DSC3 and stably transfected it to H2170 and H1299, both lacking endogenous expression of DSC3. After stable transfection, expression of DSC3 protein was detected in DSC3 transfectants c1 and c2 by western blotting.
This result was verified by immunofluorescence microscopy. As expected, membranous staining of DSC3 protein was found in DSC3 transfectants but not in mock transfected cells (Figure 4B). Overexpression of DSC3 did not cause altered expression of other desmosomal proteins such as DSC1-2, DSG1-3, desmoplakin and plakophilin, as well as other important cell-adhesion molecules β-catenin and E-cadherin (data not shown).

We assessed the effect of DSC3 on cell proliferation by determining cumulative cell number of DSC3 transfectants and mock transfectants. A reduction of cumulative cell number in DSC3 transfectants was found compared with control cells (Figure 4C). To analyze the effect of DSC3 on anchorage-independent cell growth, soft agar assay was carried out. After 4 weeks of incubation, significant reduction in colony numbers was observed in DSC3 transfectants compared with the control cells ($P < 0.05$, Figure 4D).

The effect of DSC3 on lung cancer cell motility was investigated by wound-healing assay in the presence of mitomycin C, a proliferation inhibitor. DSC3 transfectants spread along the wound edges much slower than mock transfectants (Figure 4E).

To confirm this result, transwell migration assays were carried out. As shown in Figure 4F, the number of cells that migrated into the lower chamber was significantly reduced in DSC3 transfectants compared with mock transfectants ($P < 0.01$), indicating that DSC3 inhibits tumor cell motility.

In addition, a matrigel invasion assay was performed to examine the invasive potential of the cells. As shown in Figure 4F, the number of cells that migrated through the matrigel membrane into the lower chamber was significantly reduced in DSC3 transfectants compared with mock transfectants ($P < 0.01$), suggesting that DSC3 also inhibits the invasive potential of lung cancer cells.

![Fig. 4.](image-url)
Effect of EGFR inhibition on regulation of DSC3

In SCC of head and neck, inhibition of EGFR promotes desmosome assembly by upregulation of desmosomal proteins such as desmoglein 2 and desmocollin 2 (19). To explore the effect of EGFR activation/inhibition on the expression of DSC3 in lung cancer cells, we first treated the DSC3 transfectants H2170/DSC3 c1 and c2 with EGF. Activation of EGFR led to decreased expression of DSC3 (Figure 5A). In contrast, inhibition of EGFR by gefitinib increased the expression of DSC3 (Figure 5B). Similarly, in the cell line H226 with endogenous expression of DSC3, activation of EGFR resulted in a reduction of DSC3 protein (Figure 5C).

DSC3 expression inhibits MAPK/ERK pathway in lung cancer cells

To investigate the downstream signals involved in the DSC3 mediated reduction of tumor cell growth, migration, and invasion, we examined the mitogen-activated protein kinase (MAPK)/ERK pathway. As shown in Figure 5D, DSC3 transfection resulted in reduced phosphorylation of ERK1/2. Furthermore, after cells were treated with EGF and/or PD98059, a selective inhibitor for MAPK/ERK pathway, the upregulation of phospho-ERK1/2 by EGF was greatly reduced by PD98059 (Figure 5E and Supplementary Figure S2, available at Carcinogenesis Online) and obviously the levels of phospho-ERK1/2 were higher in mock transfectants than in DSC3 transfectants after treatment of EGF and/or PD98059 simultaneously. In contrast, knockdown of DSC3 in cells with DSC3 endogenous expression (H226) or cells with exogenous expression (H2170/DSC3c1 and c2) resulted in higher level of phospho-ERK1/2 (Figure 5F and Supplementary Figure S3, available at Carcinogenesis Online). Taken together, our results indicate that DSC3 expression can inhibit the MAPK/ERK pathway.

Discussion

Numerous studies have characterized key contributions of desmosome deficiency to epithelial cancer development and progression (20,21). Our present study demonstrates that DSC3 has tumor-suppressive function possibly through inhibition of EGFR/ERK pathway in lung cancer.

We first analyzed the expression of DSC3 in lung cancer. Decreased expression of DSC3 was found in a majority of lung cancer cell lines and primary lung tumors. This result is consistent with previous studies indicating that loss or reduced expression of DSC3 is not a rare event in cancer (11,13,22). In line with the report by Tsuta et al. (15)
that 72.7% of squamous cell lung cancer expressed DSC3 protein, and DSC3 was expressed in almost all of the poorly differentiated SCC, we found that most of the SCC (63.6%) had DSC3 expression, whereas only 7.9% of adenocarcinoma exhibited DSC3 protein, suggesting that the presence of DSC3 protein is a potential biomarker for patients with lung SCC.

Next we explored the epigenetic regulation for the DSC3 gene silencing. DNA methylation contributes to the regulation of gene expression in carcinogenesis (23). In this study, we performed demethylation tests by DAC treatment. DSC3 mRNA expression was restored in 7 out of 11 lung cancer cell lines. To confirm that DSC3 downregulation was caused by the methylation of DSC3 DNA but not by the methylation of DSC3 upstream regulatory genes, BS and MSP were carried out. In line with the demethylation tests, BS and MSP showed that DSC3 promoter region was methylated in the same seven lung cancer cell lines and in a majority of primary lung tumors, demonstrating that epigenetic regulation could be at least partly responsible for DSC3 gene silencing. It is interesting to note that methylation of DSC3 DNA appeared to be a prognostic marker in patients with primary lung tumors, since patients with methylated tumors had worse overall survival compared with patients with unmethylated tumors.

The regulatory mechanisms controlling DSC3 expression are not fully understood. DSC3 is a p53 target gene and addition of wild-type p53 was found to be sufficient to induce expression of DSC3 in breast and colorectal cancers (13,24). Thus, it was of great interest to investigate the effect of p53 on DSC3 expression in lung cancer cells. We found that exogenous expression of p53 increased DSC3 expression in the cell line H2170 harboring no DSC3 DNA methylation. In the DSC3-methylated cell line H1299, induction of DSC3 expression after transfection of p53 was only achieved in the presence of the demethylating agent DAC, which was under clinical trials in patients with lung cancer (25).

To clarify the function of DSC3 in lung cancer, we constructed a DSC3 expression vector and performed stable transfection. Our in vitro studies demonstrated that re-expression of desmosomal protein DSC3 led to significantly reduced proliferative, migratory and invasive properties of lung cancer cells, indicating its tumor-suppressive activity.

The mechanisms how DSC3 inhibits tumor proliferation and invasion are not well understood. Previous studies revealed that desmosome dysfunction could promote cancer cell development through Wnt/β-catenin pathway (26–29). However, we did not observe any effect of DSC3 on Wnt/β-catenin signaling (data not shown). Various studies also demonstrated that other pathways were associated with altered desmosome. For example, loss of desmocollin 2 could confer tumorigenic phenotype to colon epithelial cells through activating Akt/β-catenin signaling (30); desmoplakin knockdown could induce the activation of ERK1/2 and AKT signaling in human keratinocytes (31). In our study, activation of EGFR led to decreased protein levels of DSC3, whereas EGFR inhibition resulted in enhanced expression of DSC3, indicating an EGFR-dependent regulation of DSC3. In the analyses of the phosphorylation levels of EGFR downstream target genes, we did not observe any alterations in PI3K, Akt and mammalian target of rapamycin (data not shown); however, we observed reduced activation levels of ERK1/2 in DSC3 transfectant cells compared with control cells. In line with this, the mitogen activated protein kinase kinase inhibitor (MEK) PD098059 reduced EGFR-dependent upregulation of phospho-ERK1/2. Moreover, knockdown of DSC3 caused enhanced levels of phospho-ERK1/2. Given the fact that MAPK (originally called ERK) signaling pathway is a major regulator of cell proliferation, migration, as well as invasion (32), we postulate that DSC3 exerts tumor-suppressive activity most probably through inactivation of the EGFR/ERK signaling pathway in human lung cancer cells.

Based on our knowledge, this is the first study reporting the tumor-suppressive function of DSC3 in cancer. The epigenetic regulation together with the mechanism of inhibition of the EGFR/ERK pathway may open new avenues for the development of cell-adhesion-based therapeutic strategy in lung cancer.

Supplementary material
Supplementary Tables 1 and 2, and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References
Desmocollin 3 in human lung cancer


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